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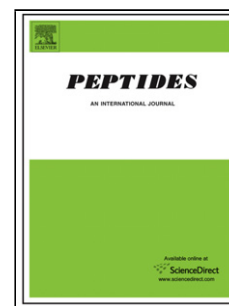
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**Purification, characterization and molecular cloning of
chymotrypsin inhibitor peptides from the venom of Burmese
*Daboia russelli siamensis***

Chun-teng Guo^{a,b}, Stephen McClean^{a*}, Chris Shaw^c, Ping-fan Rao^b, Ming-yu Ye^b,
Anthony J. Bjourson^a

^a School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine BT52
1SA, Northern Ireland, UK

^b The Institute of Biotechnology, Fuzhou University, Fuzhou 350002, Fujian Province,
People's Republic of China

^c School of Pharmacy, Queen's University, Belfast BT9 7BL, Northern Ireland, UK

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*Corresponding author: Tel: +44-2870124406. Fax: +44-2870-124965.
Email: s.mcclean@ulster.ac.uk

Abstract

One novel Kunitz BPTI-like peptide designated as BBPTI-1, with chymotrypsin inhibitory activity was identified from the venom of Burmese *Daboia russelli siamensis*. It was purified by three steps of chromatography including gel filtration, cation exchange and reversed phase. A partial N-terminal sequence of BBPTI-1, HDRPKFCYLPADPGECLAHMRSF was obtained by automated Edman degradation and a K_i value of 4.77 nM determined. Cloning of BBPTI-1 including the open reading frame and 3' untranslated region was achieved from cDNA libraries derived from lyophilized venom using a 3' RACE strategy. In addition a cDNA sequence, designated as BBPTI-5, was also obtained. Alignment of cDNA sequences showed that BBPTI-5 exhibited an identical sequence to BBPTI-1 cDNA except for an eight nucleotide deletion in the open reading frame. Gene variations that represented deletions in the BBPTI-5 cDNA resulted in a novel protease inhibitor analog. Amino acid sequence alignment revealed that deduced peptides derived from cloning of their respective precursor cDNAs from libraries showed high similarity and homology with other Kunitz BPTI proteinase inhibitors. BBPTI-1 and BBPTI-5 consist of 60 and 66 amino acid residues respectively, including 6 conserved cysteine residues. As these peptides have been reported to have influence on the processes of coagulation, fibrinolysis and inflammation, their potential application in biomedical contexts warrants further investigation.

1. Introduction

Protease inhibitors are part of the composition of Russell's viper venom, in which they exhibit varying concentrations depending on their geographical distribution [21]. Trypsin and chymotrypsin inhibitors from snake venoms are members of the functionally diverse BPTI-like (bovine pancreatic trypsin inhibitor) superfamily. The snake venom Kunitz/BPTI inhibitors are basic polypeptides with approximately 60 amino acids in which there are six cysteine residues forming three conserved disulfide bridges. They exhibit diverse functions for inhibiting serine proteinase enzymes. This superfamily is classified into two families based on protein structure; small Kunitz-type inhibitors and BPTI-like toxins and soft tick anticoagulant proteins [5]. The Kunitz BPTI proteinase inhibitor family is divisible into subgroups according to source and bioactivity. Snake Kunitz/BPTI inhibitors have been divided into non-neurotoxic and neurotoxic groups according to their functions [2]. The non-neurotoxic snake Kunitz/BPTI inhibitors include trypsin and chymotrypsin inhibitors. Another group acting as K^+ and Ca^{2+} channel blockers with little or no inhibitory activity is the neurotoxic snake Kunitz/BPTI. This group includes α -dentrotoxin, toxin K, toxin I, calcicludine, and the small subunit of β -bungarotoxin from various snake sources [11,14]. Moreover, a trypsin inhibitor occurring in a complex containing a phospholipase A2 (PLA2) and a protease has also been reported [22]. The characteristically folded domain like that of a BPTI-like peptides is also found in several proteins, for example a human Alzheimer amyloid precursor protein [17], type VI collagen alpha3(VI) [27,41], tissue factor pathway inhibitor [1] and

bikunin [38].

Although trypsin and chymotrypsin inhibitors are widely distributed in Russell's viper venom, to date only some inhibitors have been cloned. Peptides belonging to the Kunitz BPTI proteinase inhibitor family have very similar amino acid sequences, but display various functions. The present study describes the purification, properties, structure and cloning of chymotrypsin inhibitors from Burmese *Daboia russelli siamensis* venom.

2. Materials and methods

2.1 Materials

Burmese *Daboia russelli siamensis* venom was purchased from Xinyuan Jiayu management department for snake venoms (Guangzhou, China), an agency of Rainbow Snake Farm (Yingtian City, Jiangxi Province, China). A SuperdexTM Peptide 10/300 GL column was purchased from Pharmacia (Uppsala, Sweden). POROS 50HS gel was bought from Perkin Elmer (Wellesley, MA, USA). A Jupiter C₁₈ column was purchased from Phenomenex Inc. (Torrance, CA, USA). Benzoyl-D, L-4-arginine-p-nitroanilide (BAPNA) was obtained from Shanghai ShuiYuan Biotechnology Co. Ltd. (Shanghai, China). N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide was bought from Sigma (Missouri, USA). Bovine trypsin and chymotrypsin were bought from Shanghai Sheng Gong Biological Engineering and Technology Service Co. Ltd. (Shanghai, China) and Amresco (Carson, CA, USA), respectively. A Micro BCATM Protein Assay Kit was obtained from Pierce Biotechnology Inc. (Rockford, Illinois, USA). A pGEM-T Easy vector and PCR Rapid Purification Kit were purchased from Promega (Madison, WI, USA) while a DNA Sequencing Kit (BigDye Terminator v3.1) was obtained from Applied Biosystems (Foster City, California, USA). All reagents used were of analytical grade.

2.2. Isolation and Purification

2.2.1. Pretreatment of crude venom by heating

One hundred milligrams of Burmese *Daboia russelli siamensis* venom were

dissolved in 1.0 ml of 0.4/99.6 (v/v) trifluoroacetic acid (TFA)/water. The venom solution was heated at 80 °C for 30 min in a water bath. The supernatant was prepared by centrifugation at 10,000 ! g for 10 min. The precipitate was washed twice using 0.4/99.6 (v/v) TFA/water, then the supernatant was pooled and concentrated using a Christ Alpha rotating vacuum concentration centrifuge (Martin Christ, Osterode am Harz, German).

2.2.2. *SuperdexTM Peptide 10/300 GL column chromatography*

The concentrated supernatant was applied to a SuperdexTM Peptide 10/300 GL column (1.5! 30.0 cm) equilibrated with 0.02 M PBS buffer, pH 7.0, containing 0.25 M NaCl at a flow rate of 0.6 ml/min. The chromatography was conducted using a Beckman Gold HPLC system (Beckman Instruments, Brea, CA, USA) at room temperature, the eluate monitored at 214 nm using a Beckman 166 UV detector and fractions collected.2.2.3. ***POROS 50HS column chromatography***

The fraction containing chymotrypsin inhibitor activity from gel filtration chromatography was collected and diluted four-fold (V/V) with 0.02 M phosphate buffer, pH 7.0. High performance liquid chromatography (HPLC) of the diluted fractions was performed using a self-packed POROS 50HS column (0.6 ! 15.0 cm). The column was equilibrated with 0.02 M phosphate buffer, pH 7.0 at a flow rate of 1.0 ml/min. The absorbed components were eluted with a linear gradient of NaCl from 0 to 0.35 M in the equilibrium buffer for 140 min and 0.35 to 0.8 M NaCl for 100 min. The chromatography was conducted using a CCPD 8000 HPLC system from Tosoh Co. (Tokyo, Japan) at room temperature, the eluate was monitored at 214 nm with a Beckman 166 UV detector and fractions were collected.

2.2.4. Purification of BBPTI-1 on reverse phase C-18 column

The fractions with inhibitory activity against chymotrypsin from the POROS 50HS column chromatography separation were loaded onto a Jupiter C-18 column (0.46 ! 15 cm) equilibrated with 0.05/99.95 (v/v) trifluoroacetic acid (TFA)/water at a flow rate of 0.5 ml/min. The absorbed components were eluted with a linear gradient from 0.05/79.95/20 (v/v/v) TFA/water/80% acetonitrile to 0.05/65.45/34.5 (v/v/v) TFA/water/80% acetonitrile for 155 min. The chymotrypsin inhibitors were concentrated by using a ZFQ 85A rotating vacuum concentrator (Shanghai Medical Machine Ltd, China) and a Christ RVC rotating vacuum evaporator (Martin Christ, Osterode am Harz, Germany) for further characterization. POROS 50HS column chromatography was then performed as described above.

2.3. Characterization of BBPTI-1

2.3.1. Mass spectrometry

MALDI-TOF MS of positive ions of trypsin inhibitors was carried out using a Voyager DE mass spectrometer (Voyager, Applied Biosystems, Warrington, UK) operated in linear mode. Alpha-cyano-4-hydroxycinnamic acid (Sigma, UK) (10 mg/ml) was prepared in a solution containing 0.1/49.9/50 TFA/water/acetonitrile. Calibration took place using a mixture of peptides of known mass (Laser Biolabs, Sophia-Antipolis, Cedex, France).

2.3.2. Determination of peptide concentration

A Micro BCATM Protein Assay Kit (Pierce, USA) was used for determination of peptide concentrations using BSA (supplied with the kit) as the standard according to the manufacturer's protocol.

2.3.3. Amino acid sequence from automated Edman degradation

Sequence determination of purified peptide was performed by Edman degradation using a protein sequencer (Applied Biosystems Model 476A, Perkin Elmer, MA, USA). The primary structure was obtained by comparing the elution position of standard PTH-AAs.

2.3.4. Determination of chymotrypsin inhibitory activity and chymotrypsin inhibition constants (K_i)

Chymotrypsin inhibitor activities were measured according to the Mikola and Mikkonen method [28] with modifications. Assays were performed by adding together 30 !l of 5.0 ! g/ml chymotrypsin solution (Amesco, USA), 10.0 !l of sample solution, 300.0 !l of 0.2 M Tris-HCl buffer (pH 8.0) containing 0.02 M CaCl_2 and 210.0 !l of distilled water. After the mixture was preincubated at 37 °C for 20 min in a water bath, 50 !l of preheated substrate (1.5 mg/ml), N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma, USA) were added to the assay solution and the mixture was further incubated at 37 °C for 5 min. The enzymatic reaction was terminated by the addition of 60.0 !l of 30% acetic acid. Distilled water was used as the control. The mixture was centrifuged at 10,000 ! g for 5 min, and its absorbance read at 405 nm.

For the determination of the chymotrypsin inhibitor K_i value, an appropriate amount of chymotrypsin was incubated with two concentrations of substrate. Briefly, the mixture contained 50 !l of bovine chymotrypsin (5.0 ! g/ml), varying volumes of inhibitor also at a concentration of 5.0 ! g/ml, 300.0 !l of 0.2 M Tris-HCl buffer (pH 8.0) containing 0.02 M CaCl_2 and an appropriate amount of distilled water to provide a total volume of 550.0

! l. After the mixture was incubated at 37 °C for 20 min in a water bath, 50.0 ! l of preheated substrate (1.2 mg/ml or 0.6 mg/ml) were added to the assay solution for a further incubation of 5 min. The enzymatic reaction was terminated by the addition of 60.0 ! l of 30% acetic acid. References were obtained by adding acetic acid before the substrate was added to mixture. After the mixture was centrifuged at 10,000 ! g for 5 min, its absorbance was read at 405 nm. A Dixon plot [10] of $1/v$ versus I inhibitor concentration at two substrate concentrations was performed to estimate the inhibition constant and to determine if any activity was competitive or non-competitive. In such plots non-competitive activity is observed by lines converging on the x axis while for competitive activity lines converge above the x axis.

2.3.5. Detection of trypsin inhibition activity

Trypsin inhibitor activity was measured according to the Mikola and Mikkonen method [28] with modifications. Assays were performed by adding 50 ! l of trypsin solution (5.0 ! g/ml), varying concentrations of inhibitor, 300.0 ! l of 0.2 M Tris-HCl buffer (pH 7.4) containing 0.02 M CaCl_2 , and distilled water to provide a final volume of 550 ! l. After the mixture was preincubated at 37 °C for 20 min in a water bath, 50 ! l of preheated substrate (2.0 mM) (BAPNA) was added to the trypsin assay solution, and the mixture was further incubated at 37 °C for 5 min. The enzymatic reaction was terminated by the addition of 60.0 ! l of acetic acid. Distilled water was used as the control in place of protease inhibitor. After the mixture was centrifuged at 10,000 ! g for 5 min, its absorbance was read at 405 nm.

2.4. Molecular cloning of cDNA encoding chymotrypsin inhibitors

2.4.1. Construction of cDNA libraries derived from lyophilized snake venom

An mRNA isolation kit (Dynal Biotec., ASA, Oslo, Norway) was used to isolate mRNA. Briefly, 50.0 mg of lyophilized Burmese *Daboia russelli siamensis* venom was dissolved in 1.0 ml of lysis/binding buffer. Magnetized oligo-dT beads were used to isolate mRNA with polyadenylate according to manufacturer's protocol. The isolated mRNA was immediately used for a 3'-rapid amplification of cDNA ends (RACE) reaction to obtain the chymotrypsin inhibitor nucleic acid sequence based on a SMART-RACE kit (Clontech, Basingstoke, UK) method.

2.4.2. Cloning of protease inhibitors

A sense primer (P_1) sequence (5'-ATGTCTTCTGGAGGTCTTCTTCTCC-3') designed from the signal peptide was according to the B₁ chain (BPTI peptide) cDNA of a PLA₂ [37]. PCR product was obtained by utilizing a nested universal primer (NUP) (an antisense primer) from the kit and a sense primer, P_1 . PCR was performed on a thermal cycler (ThermoHybaid, Franklin, MA, USA) using the following cycle: initial denaturing at 94°C for 3 min, 40 cycles of PCR (94°C for 30s, 57°C for 30s, and 72°C for 3 min). The last cycle was 72°C for 10 min. PCR product was analyzed by agarose gel electrophoresis. The 3-RACE reaction was purified by using the Wizard[®] SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Purified PCR sample was transformed to pGEM-T Easy vector, and then cloned into JM 109 high efficiency competent cells (Promega, Madison, WI, USA) according to the protocol. Two primers

flanking the inserts in pGEM-T Easy vectors were used to amplify the inserted gene and for DNA sequencing. The inserted genes containing approximately 500 bps were selected for DNA sequencing by using a BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI 3100 automated DNA sequencer (Applied Biosystems, California, USA).

2.5. Bioinformatics of nucleotide and amino acid sequences

The AlignX programme of the Vector NTI Bioinformatics suite (Informax) was used for alignment of nucleotide sequences cloned from Burmese *Daboia russelli siamensis* venom and its similar analogs, and for alignment of amino acid sequences deduced from cloned cDNAs. Regions of local similarity between sequences were determined by BLAST searching (<http://www.ebi.ac.uk/Tools/sss/ncbiblast>).

3. Results

3.1. Purification of BBPTI-1

When Burmese *Daboia russelli siamensis* venom was dissolved in 0.4% TFA water and heated at 80°C in a water bath for 30 min, a significant amount of protein was precipitated and removed by centrifugation. Shown in Fig. 1a is the result of Superdex™ Peptide 10/300 GL column chromatography of the pretreated sample. Several peaks are shown in the chromatogram, of which one marked with an arrowhead was found to display chymotrypsin inhibition activity. The active fractions were pooled, and diluted four-fold with 0.02 M phosphate buffer, pH 7.0, then applied to a cation exchange POROS 50HS column resulting in the chromatogram shown in Fig. 1b. Nine peaks were obtained in all, of which peak 5 showed chymotrypsin inhibition activity. The fractions corresponding to peak 5 were further purified on a C-18 column to purify the active peptide, BBPTI-1 (Fig. 1c).

3.2 Properties of BBPTI-1

3.2.1. Mass spectrometry

Fig. 1d shows the MALDI-ToF mass spectrum of BBPTI-1, indicating that complete isolation of the chymotrypsin inhibitor peptide from crude venom had taken place. The mass spectral signal at 6872.81 m/z represents the singly charged state of the peptide ($[M+H]^+$) while 3438.10 m/z represents the doubly charged state ($[M+2H]^{2+}$). Deconvolution of these peaks provides masses for the peptide of 6874.20 Da as derived from the doubly charged signal and 6871.81Da from the singly charged state. The

discrepancy in mass is most likely due to the MALDI-ToF instrument operating to approximately $\pm 0.1\%$ accuracy and for this reason the mass of 6874.20 Da as derived from the doubly charged 3438.10 m/z signal is preferred as it is subject to a lower potential error.

3.2.2. Amino acid sequences of BBPTI-1

Using automated Edman degradation, the partial N-terminal amino acid sequence of BBPTI-1 was found to be HDRPKFCYLPADPGEC LAHMRSF.

3.2.3. Determination of chymotrypsin inhibition constant

The Dixon plot in Fig 2 shows that BBPTI-1 has chymotrypsin inhibition activity with a K_i value of 4.77 nM. It was competitive in its activity with that of chymotrypsin as demonstrated in Fig. 2 by lines converging above the x axis (indicated by an arrowhead).

3.2.4. Trypsin inhibition activity

BBPTI-1 strongly inhibited chymotrypsin activity, but showed no detectable inhibitory activity against trypsin.

3.3. Molecular cloning of cDNA encoding BBPTI-1 and BBPTI-5

RT-PCR product with about 500 bps was obtained from the dry venom-derived libraries of Burmese *Daboia russelli siamensis* venom. Two cDNAs of BPTI peptides designated as BBPTI-1 and BBPTI-5, were consistently cloned from Burmese *Daboia russelli siamensis* venom by using one primer designed from the signal peptide, and another primer (NUP) from the kit for PCR analysis (Fig. 3A, Fig. 3B). The cDNA

sequences of BBPTI-1 and BBPTI-5 show an open-reading frame consisted of 84 and 90 amino acid residues, respectively. Determination of the cDNA of purified BBPTI-1 was achieved by comparing the molecular mass and the N-terminal amino acid sequence of purified BBPTI-1 and the peptide sequence deduced from cDNA. In terms of molecular weight both are very similar. Assuming that cysteines are oxidized, the deduced cDNA sequence has an average molecular weight of 6873.79Da while the molecular weight of the mature peptide as determined by MALDI-ToF mass spectrometry using the doubly charged signal (3438.10m/z) shown in Fig. 1d is 6874.20 Da. These two values are in very close agreement and demonstrate that oxidation of cysteines to form disulfide bonds has indeed taken place.

3.4 Comparison of nucleotide and amino acid sequences and homology research

Alignment of nucleotide sequences of BBPTI-1, BBPTI-5, CBPTI-3 from Chinese *Daoboia russellii russellii* venom and IBPTI-III from Indian *Daoboia russellii russellii* venom (Fig. 4A) was performed by using the AlignX programme of the Vector NTI Bioinformatics suite (Life Technologies, UK). This revealed that these peptides have a high degree of similarity in nucleotide sequences in the translated signal peptide and 3' untranslated regions. An obvious gene deletion compared to BBPTI-1 cDNA was found in the BBPTI-5 nucleotide sequence open-reading frame, which resulted in changing the site of the termination codon and producing BBPTI-5 with 66 amino acid residues.

Alignment of BBPTI-1, BBPTI-5, CBPTI-3 and IBPTI-3 open-reading frame amino acid sequences (Fig. 4B) reveals they have a high degree of homology; in particular their

signal peptide sequences are identical. Fig. 4A shows that BBPTI-1 contains 60 amino acids with 6 cysteine residues, whose positions in the amino acid sequences are conserved in the four peptides investigated.

4. Discussion

In snake venoms, there are many serine protease inhibitors belonging to the BPTI-like superfamily. To date a number of BPTI-like superfamily serine protease inhibitors from Viperidae and Elapidae venoms have been purified or characterized [3,4,12,13,15,16,18,19,25,26,29-31,32-34,35,36,39]. In this paper, one chymotrypsin inhibitor, BBPTI-1, was purified to homogeneity from the venom of Burmese *Daboia russelli siamensis* by gel filtration, cation exchange and reversed phase chromatography. The purified BBPTI-1 is basic and heat stable, which has been elucidated by the procedures of preparing venom samples and subsequent purification. The precursor cDNAs of BBPTI-1 and unknown BBPTI-5 have been cloned from libraries derived from Burmese *Daboia russelli siamensis* venom according to a method developed in our laboratory [6].

The venom of Burmese *Daboia russelli siamensis* was heated at 80°C for 30 min, which resulted in the majority of protein being precipitated, so the purification procedures of BBPTI-1 became simpler. The molecular weight of BBPTI-1 is 6874 Da, (Fig. 1d) and is composed of 60 amino acid residues. BBPTI-1 showed competitive chymotrypsin inhibitory activity, but no detectable trypsin inhibitory activity. Generally, chymotrypsin inhibitors exhibit no inhibitory trypsin activity, as is the case with Oh11-1 from *Ophiophagus hannah* venom [3], CBPTI-3 from Chinese *Daboia russellii siamensis* [13] and NA-CI from *Naja atra* venom [39], but Ritonja reported a chymotrypsin inhibitor that inhibited trypsin and human kallikrein weakly [31]. The

dissociation constant, K_i value of BBPTI-1 is similar to that reported for a chymotrypsin inhibitor from *Vipera ammodytes* venom of 4.3 nM [31], higher than CBPTI-3 from Chinese *Daoia russullii siamensis* venom, 2.55nM [13], but lower than the reported value for Oh11-1 from *Ophiophagus hannah* venom, 3.52 μ M [3] and NA-CI from *Naja atra* venom, 25 nM [39].

The molecular weight of the mature BBPTI-1 peptide deduced from the cDNA sequence is 6873.79 Da (<http://www.peptidesynthetics.co.uk/tools/>) assuming that cysteines are oxidized. This is in close agreement to the observed mass (6874.20Da) derived from MALDI-TOF mass spectrometry of the doubly charged signal (Fig. 1d). A comparison of cDNA of BBPTI-1, BBPTI-5, CBPTI-3 and IBPTI-III showed identical sequences in their signal peptide (Fig. 4B) and high similarity in the 3'-noncoding regions. A gene deletion compared to BBPTI-1 cDNA was found close to the 3'-terminal in BBPTI-5 cDNA, which resulted in a mature peptide with 66 amino acid residues. Following NCBI-BLAST (<http://www.ebi.ac.uk/Tools/sss/ncbiblast/>) mature peptide homology searching, 6 amino acid differences were noted for BBPTI-1 compared to CBPTI-3 and 7 amino acid differences compared to IBPTI-3, especially for the P₁ position; in BBPTI-1 this is Leu instead of Met in CBPTI-3 and IBPTI-3 (Fig. 4B). BBPTI-1 showed 90% similarity to CBPTI-3 (54/60) and 88% to IBPTI-3 (53/60). *Naja naja atra* chymotrypsin inhibitor (NACI) genes [8] and several B chain genes of β -bungarotoxins have been reported by Cheng et al. [7], and they share virtually an identical structural organization containing 3 exons and 2 introns, and a high degree of

sequence identity. This suggests that Kunitz/BPTI protease inhibitors and B chains may have originated from a common ancestor, as was also elucidated by comparing signal peptide amino acid sequences of snake Kunitz/BPTI proteins and obtain highly conserved sequences [40]. In this paper, highly conserved signal peptide sequences of Kunitz/BPTI proteins were also found (Fig. 4B). Moreover, by analyzing the protein sequences of the snake Kunitz/BPTI family, the progressive development of this family has been achieved by gene duplication followed by diversification [7,40].

With regard to the competitive inhibition activity study, the P₁ residue in the center of the binding loop is particularly important and its variants have been shown to greatly alter the specificity and potential activity of these peptides [9,20]. The P₁ amino acid in many inhibitor families shows a high degree of variability [24]. Cardle and Dufton [2] analyzed the Kunitz BPTI proteinase inhibitor sequences for each subgroup containing different functions and revealed that amino acid side chains at interactive sites were conserved. A typical trypsin inhibitor has a positively charged Arg or Lys residue at P₁, whereas a chymotrypsin inhibitor has a large hydrophobic Phe, Leu, Met, Tyr, or Trp residue [23]. Two chymotrypsin inhibitors containing Asn at P₁ were also found in *Bungarus fasciatus* venom [25] and *Ophiophagus hannah* venom [3]. In this study the P₁ amino acid residue of BBPTI-1 and BBPTI-5 is leucine (Fig. 4B), providing further confirmation that these peptides are chymotrypsin inhibitors.

Snake venom Kunitz/BPTI inhibitors are considered to have influence on the processes of coagulation, fibrinolysis and inflammation [33] and protease inhibitors such

as the polypeptides aprotinin and ulinastatin, have been used in pharmacological contexts.

In this paper the properties, structure and cloning of chymotrypsin inhibitors from Burmese *Daboia russelli siamensis* venom have been described.

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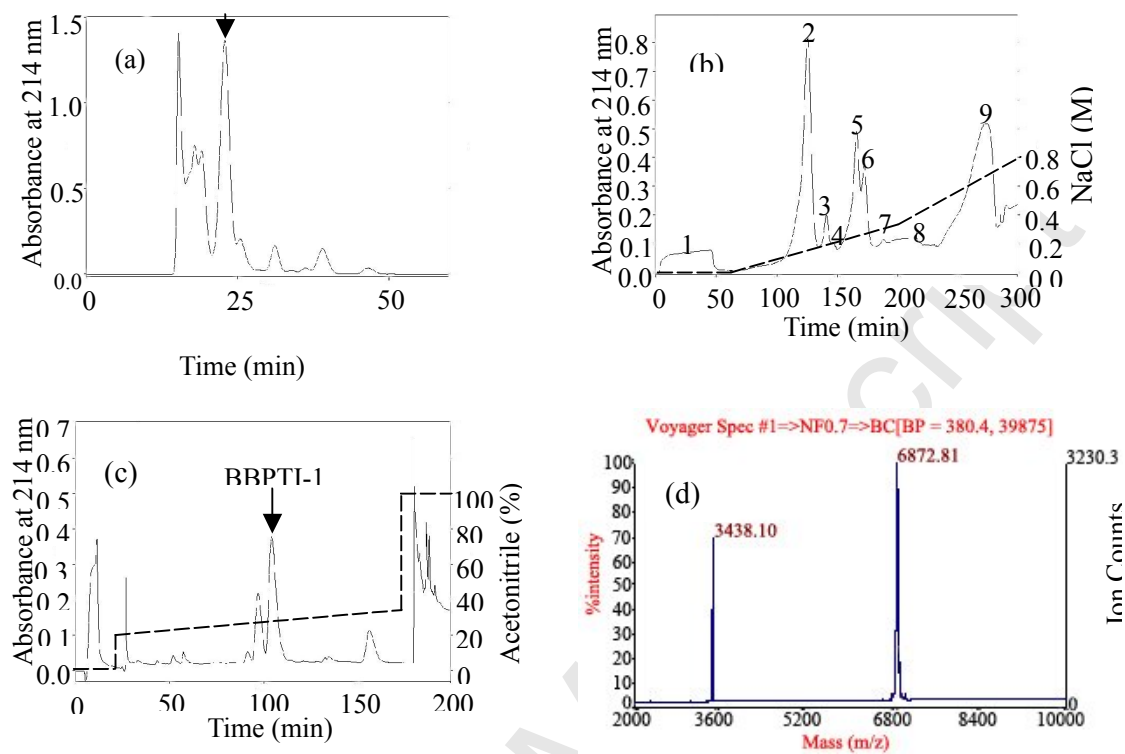


Fig. 1

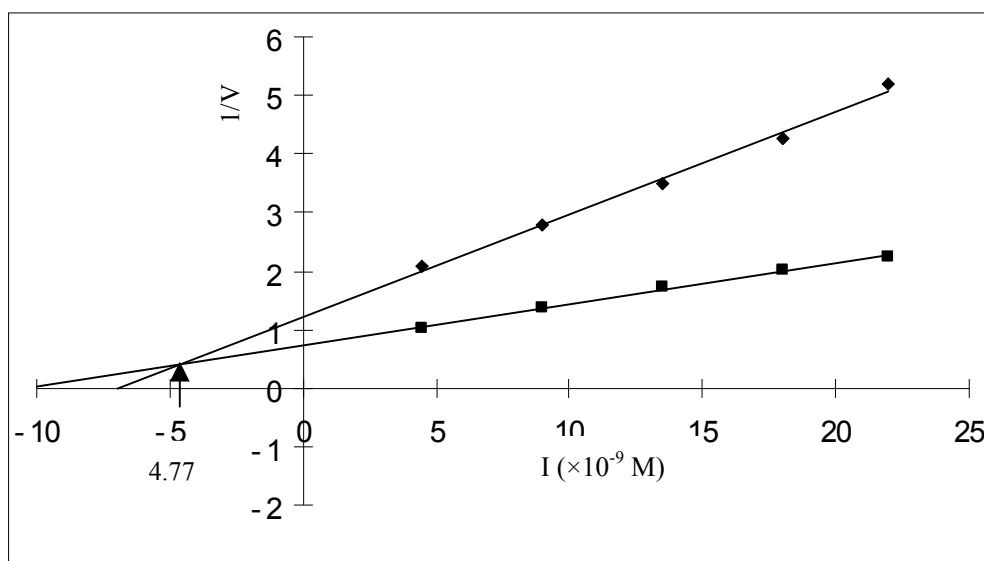


Fig. 2

(A)

M S S G G L L L L L G L L T L W A .
 1 ATGTCTTCTG GAGGTCTTCT TCTCCTGCTG GGA CTCTCA CCCTCTGGGC

 . E L T P I S G H D R P K F C Y L P .
 51 AGAGCTGACC CCCATCTCCG GCCACGACCG TCCAAAGTTT TGTATCTCC

. A D P G E C L A H M R S F Y Y D
 101 CTGCTGATCC TGGAGAATGT TTGGCCATA TCGTAGTTT CTACTACGAC

 S E S K K C K E F I Y G G C H G N .
 151 TCGGAATCAA AGAAATGTAA AGAATTTATT TACGGTGGAT GCCATGGAA

 . A N K F P S R D K C R Q T C G G K .
 201 TGCCAACAAG TTCCGAGCA GGGATAAATG TCGCCAGACC TGTGGTGGTA

 . *
 251 AGTAGCATCC GCAAAGGGA GACCCACCTG AATTGGGTCT AATTCGCCAA

 301 CTTCACTGAG AGGCGTTCCT TCTGTCCTGG ATTATTCTGG AGACCCTCCC
 351 CCCAAACCCG CCCTGGCTTC ATCCCTTCTG TTCTGCAATA AAGCTTTGTT
 401 CTCGCTGCAA AAAAAAAAAA AAAAAAAAAA

(B)

M S S G G L L L L L G L L T L W A .
 1 ATGTCTTCTG GAGGTCTTCT TCTCCTGCTG GGA CTCTCA CCCTCTGGGC

 . E L T P I S G H D R P K F C Y L P .
 51 AGAGCTGACC CCCATCTCCG GCCACGACCG TCCAAAGTTT TGTATCTCC

. A D P G E C L A H M R S F Y Y D

101 CTGCTGATCC TGGAGAATGT TTGGCCCATA TGCCTAGTTT CTACTACGAC

 S E S K K C K E F I Y G G C H G N .
 151 TCGGAATCAA AGAAATGTAA AGAATTTATT TACGGTGGAT GCCATGGGAA

 . A N K F P S R D K C R Q T C G A S .
 201 TGCCAACAAG TTTCCGAGCA GGGATAAATG TCGCCAGACC TGTGGTGCAT

 . A K G R P T *
 251 CCGCAAAGGG GAGACCCACC TGAATTGGGT CTAATTCGCC AACTTCACTG

 301 AGAGGCGTTC CTTCTGTCCT GGATTATTCT GGAGACCCTC CCCCCAAACC

 351 CGCCCTGGCT TCATCCCTTC TGTTCTGCAA TAAAGCTTTG TTCTCGCTGC

 401 AAAAAAAAAA AAAAAAAAAA AAAAAA

Fig. 3

(A)

		1	50
BBPTI-1	(1)	ATGTCTTCTGGAGGTCTTCTTCTCCTGCTGGGACTCCTCACCCTCTGGGC	
BBPTI-5	(1)	ATGTCTTCTGGAGGTCTTCTTCTCCTGCTGGGACTCCTCACCCTCTGGGC	
CBPTI-3	(1)	ATGTCTTCTGGAGGTCTTCTTCTCCTGCTGGGACTCCTCACCCTCTGGGC	
IBPTI-III	(1)	ATGTCTTCTGGAGGTCTTCTTCTCCTGCTGGGACTCCTCACCCTCTGGGC	
		51	100
BBPTI-1	(51)	AGAGCTGACCCCCATCTCCGGCCACGACCGTCCAAAGTTTTGTTATCTCC	
BBPTI-5	(51)	AGAGCTGACCCCCATCTCCGGCCACGACCGTCCAAAGTTTTGTTATCTCC	
CBPTI-3	(51)	AGAGCTGACCCCCATCTCCGGCCACGACCGTCCAAAGTTTTGTTATCTCC	
IBPTI-III	(51)	AGAGCTGACCCCCATCTCCGGCCACGACCGTCCAAAGTTTTGTTATCTCC	
		101	150
BBPTI-1	(101)	CTGCTGATCCTGGAGAATGTATTGGCCCATATCCGTAGTTTCTACTACGAC	
BBPTI-5	(101)	CTGCTGATCCTGGAGAATGTATTGGCCCATATCCGTAGTTTCTACTACGAC	
CBPTI-3	(101)	CTGCTGATCCTGGAGAATGTATTGGCCCATATCCGTAGTTTCTACTACGAC	
IBPTI-III	(101)	CTGCTGATCCTGGAGAATGTATTGGCCCATATCCGTAGTTTCTACTACGAC	
		151	200
BBPTI-1	(151)	TCGGAATCAAAGAAATGTAAAGAATTTATTTACGGTGGATGCCATGGGAA	
BBPTI-5	(151)	TCGGAATCAAAGAAATGTAAAGAATTTATTTACGGTGGATGCCATGGGAA	
CBPTI-3	(151)	TCGGAATCAAAGAAATGTAAAGAATTTATTTACGGTGGATGCCATGGGAA	
IBPTI-III	(151)	TCGGAATCAAAGAAATGTAAAGAATTTATTTACGGTGGATGCCATGGGAA	
		201	250
BBPTI-1	(201)	TGCCAACAAGTTTCCGAGCAGGGATAAATGTCGCCAGACCTGTGGTGGTA	

BBPTI-5 (201) TGCCAACAAGTTTCCGAC CAGGGATAAATGTCGCCAGACCTGTGGT-----

CBPTI-3 (201) TGCCAACAATTTTCCGAC CAGGGATAAATGTCGCCAGACCTGTCTGGTA

IBPTI-III (201) TGCCAACAATTTTCCGAC CAGGGATAAATGTCGCCAGACCTGTCTGGTA

251 300

BBPTI-1 (251) AGTAGCATCCGC AAAGGGGAGACC CACCTGAATTGGTCTAATTCGCCAA

BBPTI-5 (247) ----GCATCCGC AAAGGGGAGACC CACCTGAATTGGTCTAATTCGCCAA

CBPTI-3 (251) AGTAGCACCCAG AAAGGGGAGACA CACCTGAATTGGTCTAATTCGCCAA

IBPTI-III (251) AGTAGCACCCAG AAAGGGGAGACA CACCTGAATTGCATCTAATTCGCCAA

301 350

BBPTI-1 (301) CTTCACTGAGAGGCGTTCTTCTGTCTGGATTATTCTGGAGACCCTCCC

BBPTI-5 (293) CTTCACTGAGAGGCGTTCTTCTGTCTGGATTATTCTGGAGACCCTCCC

CBPTI-3 (301) CTTCACTGAGAGGCGTTCTTCTGTCTGGATTATTCTGGAGACCCTCCC

IBPTI-III (301) CTTCACTGAGAGGCGTTCTTCTGTCTGGATTATTCTGGAGTCCCTCCC

351 400

BBPTI-1 (351) CCCAAACCCGCCCTGGCTTCATCCCTTCTGTTCTGCAATAAAGCTTTGTT

BBPTI-5 (343) CCCAAACCCGCCCTGGCTTCATCCCTTCTGTTCTGCAATAAAGCTTTGTT

CBPTI-3 (351) CCCAAACCCGCCCTGGCTTCATCCCTTCTGTTCTGCAATAAAGCTTTGTT

IBPTI-III (351) CGCAAACCCGCCCTGGCTTCATCCCTTCTGTTCTGCAATAAAGCTTTGTT

401 435

BBPTI-1 (401) CTCGCTGCAAAAAAAAAAAAAAAAAAAAAA-----

BBPTI-5 (393) CTCGCTGCAAAAAAAAAAAAAAAAAAAAAA

CBPTI-3 (401) CTCGCTGCAAAAAAAAAAAAAAAAAAAAAA-----

IBPTI-III (401) CTCGCTGCAAAAAAAAAAAAAAAAAAAAA-----

(B)

1 50 ↓

BBPTI-1 (1) MSSGGLLLLLGLLTLWAE LTPISGHDRPKFCYLPADPGECLAHMRSFYYD

BBPTI-5 (1) MSSGGLLLLLGLLTLWAE LTPISGHDRPKFCYLPADPGECLAHMRSFYYD

CBPTI-3 (1) MSSGGLLLLLGLLTLWAE LTPISGHDRPKFCYLPADPGECLAHMRSFYYD

IBPTI-III (1) MSSGGLLLLLGLLTLWAE LTPISGHDRPKFCYLPADPGECLAHMRSFYYD

51 90

BBPTI-1 (51) SESKKCKEFYGGCHGNANFPSTRDKCRQTCGGK-----

BBPTI-5 (51) SESKKCKEFYGGCHGNANFPSTRDKCRQTCGASAKGRPT

CBPTI-3 (51) SESKKCKEFYGGCHGNANFPSTRDKCRQTCRGK-----

IBPTI-III (51) SESKKCKEFYGGCHGNANFPSTRDKCRQTCRGK-----

Fig. 4

Fig. 1. Chromatographic and mass spectrometric profiles of BBPTI-1 purified from the venom of Burmese *Daboia russellii siamensis*. (a) Size exclusion HPLC profile of prepared Burmese *Daboia Russelii siamensis* venom. (b) Poros 50 HS cation exchange column purification of protease inhibitor fraction from size exclusion chromatography. (c) HPLC profile of components displaying chymotrypsin inhibition on a reversed phase C-18 column. (d) MALDI-TOF mass spectrum of purified BBPTI-1.

Fig. 2 Dixon plot showing inhibitory effects of BBPTI-1 on chymotrypsin activity on N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide in the presence of substrate at concentrations of 0.08 mM and 0.16 mM, respectively. Convergence of the lines above the x axis shows competitive activity (indicated by an arrowhead).

Fig. 3. cDNAs and deduced amino acid sequences of BBPTI-1 (A) and BBPTI-5 (B) precursors. Mature peptide sequence are denoted in bold. The sequence underlined was confirmed by automated Edman degradation. A stop code is indicated by an asterisk.

Fig. 4. Alignment of nucleotide sequences (A) and amino acid sequences (B) of BBPTI-1 (GenBank accession no. GenBank: AM411368), BBPTI-5 (GenBank accession no. GenBank: AM411372), CBPTI-3 (GenBank accession no. GenBank: AM411363) and IBPTI-III (GenBank accession no. GenBank: DQ365980). Identical bases and amino acids in all sequences are shaded in black while similar base and amino acid sequences are shaded in grey or in white. The arrow indicates the critical residue (site P₁) of the antiprotease loop. Gaps are inserted for optimal alignment.

Highlights

- ▶ A novel chymotrypsin inhibitor, BBPTI-1, was found in Burmese *Daboia russellii siamensis* venom
- ▶ BBPTI-1 showed strong chymotrypsin inhibition activity, but no trypsin inhibition activity
- ▶ BBPTI-1 was cloned along with another cDNA with an 8 nucleotide deletion